

*Application
for
United States Letters Patent*

To all whom it may concern:

Be it known that **Taka-Aki Sato**

have invented certain new and useful improvements in

**METHOD OF PREPARING A PROTEIN ARRAY BASED ON
BIOCHEMICAL PROTEIN-PROTEIN INTERACTION**

of which the following is a full clear and exact description.

10092133.030002

**METHOD OF PREPARING A PROTEIN ARRAY BASED ON
BIOCHEMICAL PROTEIN-PROTEIN INTERACTION**

5 The subject matter disclosed herein was made with
Government support under Grant No. R01GM55147-01 from the
National Institutes of Health of the United States
Department of Health and Human Services. Accordingly, the
10 U.S. Government may have certain rights in this
application.

BACKGROUND

15 Throughout this application, various publications are
referenced by author and date. Full citations for these
publications may be found listed alphabetically at the end
of the specification immediately preceding Sequence Listing
and the claims. The disclosures of these publications in
their entireties are hereby incorporated by reference into
20 this application in order to more fully describe the state
of the art as known to those skilled therein as of the date
of the invention described and claimed herein.

25 Fas (APO-1/CD95) and its ligand have been identified as
important signal-mediators of apoptosis (Itoh, et al. 1991)
The structural organization of Fas (APO-1/CD95) has
suggested that it is a member of the tumor necrosis factor
receptor superfamily, which also includes the p75 nerve
growth factor receptor (NGFR) (Johnson, et al. 1986), the
30 T-cell-activation marker CD27 (Camerini, et al. 1991), the
Hodgkin-lymphoma-associated antigen CD30 (Smith, et al.
(1993), the human B cell antigen CD40 (Stamenkovic, et al.
1989), and T cell antigen OX40 (Mallett, et al. 1990).
Genetic mutations of both Fas and its ligand have been
35 associated with lymphoproliferative and autoimmune
disorders in mice (Watanabe-Fukunaga, et al. 1992;
Takahashi, et al. 1994). Furthermore, alterations of Fas
expression level have been thought to lead to the induction

10092138 030602
200005 83126001

of apoptosis in T-cells infected with human immunodeficiency virus (HIV) (Westendorp, et al. 1995).

Several Fas-interacting signal transducing molecules, such as Fas-associated phosphatase-1 (FAP-1) (Figure 1) (Sato, et al. 1995), FADD/MORT1 (Chinnaiyan, et al. 1995; Boldin, et al. 1995; Kischkel, et al. 1995) and RIP (Stanger, et al. 1995), have been identified using yeast two-hybrid and biochemical approaches. All but FAP-1 associate with the functional cell death domain of Fas and overexpression of FADD/MORT1 or RIP induces apoptosis in cells transfected with these proteins. In contrast, FAP-1 is the only protein that associates with the negative regulatory domain (C-terminal 15 amino acids) (Ito, et al. 1993) of Fas and that inhibits Fas-induced apoptosis.

FAP-1 (PTPN13) has several alternatively-spliced forms that are identical to PTP-BAS/hPTP1E/PTPL1, (Maekawa, et al. 1994; Banville, et al. 1994; Saras, et al. 1994) and contains a membrane-binding region similar to those found in the cytoskeleton-associated proteins, ezrin, (Gould et al. 1989) radixin (Funayama et al. 1991) moesin (Lankes, et al. 1991), neurofibromatosis type II gene product (NFII) (Rouleau, et al. 1993), and protein 4.1 (Conboy, et al. 1991), as well as in the PTPases PTPH1 (Yang, et al. 1991), PTP-MEG (Gu, et al. 1991), and PTPD1 (Vogel, et al. 1993). FAP-1 intriguingly contains six GLGF (PDZ/DHR) repeats that are thought to mediate intra-and inter-molecular interactions among protein domains. The third GLGF repeat of FAP-1 was first identified as a domain showing the specific interaction with the C-terminus of Fas receptor (Sato, et al. 1995). This suggests that the GLGF domain may play an important role in targeting proteins to the submembranous cytoskeleton and/or in regulating biochemical activity. GLGF repeats have been previously found in

10092138.030602

guanylate kinases, as well as in the rat post-synaptic density protein (PSD-95) (Cho, et al. 1992), which is a homolog of the *Drosophila* tumor suppressor protein, lethal-(1)-disc-large-1 [*dlg-1*] (Woods, et al 1991; Kitamura, et al. 1994). These repeats may mediate homo- and hetero-dimerization, which could potentially influence PTPase activity, binding to Fas, and/or interactions of FAP-1 with other signal transduction proteins. Recently, it has also been reported that the different PDZ domains of proteins interact with the C-terminus of ion channels and other proteins (Figure 1) (TABLE 1) (Kornau, et al. 1995; Kim, et al. 1995; Matsumine, et al. 1996).

TABLE 1. Proteins that interact with PDZ domains.

Protein	C-terminal sequence	Associated protein	Reference
Fas (APO-1/CD95)	SLV	FAP-1	2
NMDA receptor NR2 subunit	SDV	PSD95	3
Shaker-type K ⁺ channel	TDV	PSD95 & DLG	4
APC	TEV	DLG	5

A recent trend in biology, biotechnology and medicine is the use of arrays of immobilized biological compounds in studies of immunoassays and enzymatic reactions (see Mendoza, et al. 1999; Arenkov, et al. 2000). For example, mass-sensing, multianalyte microarray immunoassays have been performed (Rowe, et al. 1998; Silzel, et al. 1998). The use of arrays allows for large scale and high-throughput studies of multiple samples in parallel. Integration of microarray technology into the experimental methodology also may increase efficiency in many instances, such as through reducing the volume of samples and reagents required.

It would be desirable to have high-throughput and low cost methodologies for preparing protein arrays based on protein-protein interaction, and which keep the proteins in a functionally active state and allow, for example, multiple drug screenings under physiological conditions.

SUMMARY OF THE INVENTION

This disclosure provides a method of preparing a protein array based on biochemical protein-protein interaction, comprising (a) depositing on a substrate an array of a first protein, the first protein comprising a PDZ domain, and (b) applying a second protein, which comprises an amino acid sequence (S/T)-X-(V/I/L)-COOH, to the first protein array, the amino acid sequence (S/T)-X-(V/I/L)-COOH of the second protein binding to the PDZ domain of the first protein, wherein each hyphen represents a peptide bond, each parenthesis encloses amino acids which are alternatives to one other, each slash within such parentheses separates the alternative amino acids, and the X represents any amino acid which is selected from the group comprising the twenty naturally occurring amino acids.

This disclosure also provides a method of preparing a protein array, (a) depositing on a substrate an array of first proteins, each first protein comprising a corresponding PDZ domain, and (b) applying a second protein, which comprises an amino acid sequence (S/T)-X-(V/I/L)-COOH, to the array of first proteins, the amino acid sequence (S/T)-X-(V/I/L)-COOH of the second protein, for each of the first proteins, binding to the PDZ domain of the first protein, wherein each hyphen represents a peptide bond, each parenthesis encloses amino acids which are alternatives to one other, each slash within such parentheses separates the alternative amino acids, and the

X represents any amino acid which is selected from the group comprising the twenty naturally occurring amino acids.

5 This disclosure also provides a method of preparing a protein array, (a) depositing on a substrate an array of a first protein, the first protein comprising a PDZ domain, and (b) applying a plurality of second proteins, each of which comprises a corresponding amino acid sequence (S/T)-
10 X-(V/I/L)-COOH, to corresponding elements of the first protein array, for each of the second proteins, the amino acid sequence (S/T)-X-(V/I/L)-COOH of the second protein binding to the PDZ domain of the first protein in the corresponding array element, wherein each hyphen represents
15 a peptide bond, each parenthesis encloses amino acids which are alternatives to one other, each slash within such parentheses separates the alternative amino acids, and the X represents any amino acid which is selected from the group comprising the twenty naturally occurring amino
20 acids.

This disclosure also provides a method of preparing a protein array, comprising (a) depositing on a substrate an array of a first polypeptide, the first polypeptide
25 comprising a PDZ domain, and (b) applying a second polypeptide which comprises an amino acid sequence (S/T)-X-(V/I/L)-COOH to the first polypeptide array, the amino acid sequence (S/T)-X-(V/I/L)-COOH of the second polypeptide binding to the PDZ domain of the first polypeptide, wherein
30 each hyphen represents a peptide bond, each parenthesis encloses amino acids which are alternatives to one other, each slash within such parentheses separates the alternative amino acids, and the X represents any amino acid which is selected from the group comprising the twenty
35 naturally occurring amino acids.

10092138.030602

BRIEF DESCRIPTION OF THE FIGURES

Figure 1. Diagram of Fas-associated phosphatase-1 protein, showing the six GLGF (PDZ/DHR) domain repeats; comparison of similar membrane binding sites with other proteins and proteins that contain GLGF (PDZ/DHR) repeats.

Figures 2A, 2B, 2C and 2D. Mapping of the minimal region of the C-terminal of Fas required for the binding to FAP-1. Numbers at right show each independent clone (Figures 2C and 2D).

2A. Strategy for screening of a random peptide library by the yeast two-hybrid system.

2B. Alignment of the C-terminal 15 amino acids of Fas between human (Sequence I.D. No. 5), rat (Sequence I.D. No. 6), and mouse (Sequence I.D. No. 7).

2C. The results of screening a semi-random peptide library. Top row indicates the amino acids which were fixed based on the homology between human and rat. Dash lines show unchanged amino acids.

2D. The results of screening a random peptide library (Sequence I.D. No. 8, Sequence I.D. No. 9, Sequence I.D. No. 10, Sequence I.D. No. 11, Sequence I.D. No. 12, Sequence I.D. No. 13, Sequence I.D. No. 14, Sequence I.D. No. 15, Sequence I.D. No. 16, Sequence I.D. No. 17, respectively).

Figures 3A, 3B and 3C. Inhibition assay of Fas/FAP-1 binding *in vitro*.

3A. Inhibition assay of Fas/FAP-1 binding using the C-terminal 15 amino acids of Fas. GST-Fas fusion protein (191-355) was used for *in vitro* binding assay (lane 1, 3-10). GST-Fas fusion protein (191-320) (lane 2) and 1 mM human PAMP (N-terminal 20 amino acids of proadrenomedullin, M.W. 2460.9) (lane 3) were used as

negative controls. The concentrations of the C-terminal 15 amino acids added were 1 (lane 4), 3 (lane 5), 10 (lane 6), 30 (lane 7), 100 (lane 8), 300 (lane 9), and 1000 μ M (lane 10).

- 5 3B. Inhibition assay of Fas/FAP-1 binding using the truncated peptides corresponding to the C-terminal 15 amino acids of Fas. All synthetic peptides were acetylated for this inhibition assay (Sequence I.D. No. 4, Sequence I.D. No. 18, Sequence I.D. No. 19, Sequence I.D. No. 20, Sequence I.D. No. 21, Sequence I.D. No. 22, Sequence I.D. No. 23, respectively).

10 3C. Inhibitory effect of Fas/FAP-1 binding using the scanned tripeptides.

15 **Figures 4A, 4B, 4C and 4D.**

4A. Interaction of the C-terminal 3 amino acids of Fas with FAP-1 in yeast.

4B. Interaction of the C-terminal 3 amino acids of Fas with FAP-1 in vitro.

20 4C. Immuno-precipitation of native Fas with GST-FAP-1.

4D. Inhibition of Fas/FAP-1 binding with Ac-SLV or Ac-SLY.

Figures 5A, 5B, 5C, 5D, 5E and 5F. Microinjection of Ac-SLV into the DLD-1 cell line. Triangles identify the cells both that were could be microinjected with Ac-SLV and that showed condensed chromatin identified. On the other hand, only one cell of the area appeared apoptotic when microinjected with Ac-SLY.

25 5A. Representative examples of the cells microinjected with Ac-SLV in the presence of 500 ng/ml CH11 are shown in phase contrast.

30 5B. Representative examples of the cells microinjected with AC-SLY in the presence of 500 ng/ml CH11 are shown in phase contrast.

10092138.030602

5C. Representative examples of the cells microinjected with Ac-SLV in the presence of 500 ng/ml CH11 are shown stained with FITC.

5 5D. Representative examples of the cells microinjected with AC-SLY in the presence of 500 ng/ml CH11 are shown stained with FITC.

10 5E. Representative examples of the cells microinjected with Ac-SLV in the presence of 500 ng/ml CH11 are shown with fluorescent DNA staining with Hoechst 33342.

5F. Representative examples of the cells microinjected with AC-SLY in the presence of 500 ng/ml CH11 are shown in fluorescent DNA staining with Hoechst 33342.

15 **Figure 6. Quantitation of apoptosis in microinjected DLD-1 cells.**

Figures 7A, 7B, 7C, 7D, 7E, 7F, 7G, and 7H.

20 7A. Amino acid sequence of human nerve growth factor receptor (Sequence I.D. No. 24).

7B. Amino acid sequence of human CD4 receptor (Sequence I.D. No. 25).

25 7C. The interaction of Fas-associated phosphatase-1 to the C-terminal of nerve growth factor receptor (NGFR) (p75).

7D. Amino acid sequence of human colorectal mutant cancer protein (Sequence I.D. No. 26).

7E. Amino acid sequence of protein kinase C, alpha type (Sequence I.D. No. 27).

30 7F. Amino acid sequence of serotonin 2A receptor (Sequence I.D. No. 28).

7G. Amino acid sequence of serotonin 2B receptor (Sequence I.D. No. 29).

35 7H. Amino acid sequence of adenomatosis polyposis coli protein (Sequence I.D. No. 30).

20090322001

Figure 8. Representation of the structural characteristics of p75 NGFR (low-affinity nerve growth factor receptor).

5 Figure 9. Comparison of the C-terminal ends of Fas and p75 NGFR.

10 Figure 10. In vitro interaction of ³⁵S-labeled FAP-1 with various receptors expressed as GST fusion proteins. The indicated GST fusion proteins immobilized on glutathione-Sepharose beads were incubated with in vitro translated, ³⁵S-labeled FAP-1 protein. After the beads were washed, retained FAP-1 protein was analyzed by SDS-PAGE and autoradiography.

15 Figures 11A and 11B. In vitro interaction ³⁵S-labeled FAP-1 with GST-p75 deletion mutants.

20 11A. Schematic representation of the GST fusion proteins containing the cytoplasmic domains of p75 and p75 deletion mutants. Binding of FAP-1 to the GST fusion proteins with various p75 deletion mutants is depicted at the right and is based on data from (2B).

25 11B. Interaction of in vitro translated, ³⁵S-labeled FAP-1 protein with various GST fusion proteins immobilized on glutathione-Sepharose beads. After the beads were washed, retained FAP-1 protein was analyzed by SDS-PAGE and autoradiography.

30

Figure 12. The association between LexA-C-terminal cytoplasmic region of p75NGFR and VP16-FAP-1. The indicated yeast strains were constructed by transformation and the growth of colonies was tested. +/- indicates the

growth of colonies on his⁻ plate.

5 **FIGS. 13A-13C** provide schematic representations of a method of preparing a protein array based on biochemical protein-protein interaction, according to an embodiment of the present disclosure.

10 **FIGS. 14A-14C** provide schematic representations of some exemplary types of substrates that may be used for preparing a protein array based on biochemical protein-protein interaction.

DETAILED DESCRIPTION

15 As used herein, amino acid residues are abbreviated as follows: A, Ala; C, Cys; D, Asp; E, Glu; F, Phe; G, Gly; H, His; I, Ile; K, Lys; L, Leu; M, Met; N, Asn; P, Pro; Q, Gln; R, Arg; S, Ser; T, Thr; V, Val; W, Trp; and Y, Tyr.

20 In order to facilitate an understanding of the material which follows, one may refer to Sambrook, et al., 1989 for certain frequently occurring methods and/or terms which are described therein.

25 The present disclosure provides a method of preparing a protein array based on biochemical protein-protein interaction, comprising the steps of: (a) depositing on a substrate an array of a first protein, the first protein comprising a PDZ domain; and (b) applying a second protein, which comprises an amino acid sequence (S/T)-X-(V/I/L)-COOH, to the first protein array, the amino acid sequence (S/T)-X-(V/I/L)-COOH of the second protein binding to the PDZ domain of the first protein. Each hyphen represents a peptide bond, each parenthesis encloses amino acids which are alternatives to one other, each slash within such
35 parentheses separates the alternative amino acids, and the

1009213-030602

X represents any amino acid which is selected from the group comprising the twenty naturally occurring amino acids.

- 5 The amino acid sequence (S/T)-X-(V/I/L) may be fused to the C-terminal of the second protein.

The protein array may be maintained under physiological condition, and used to screen one or more drug targets.

10

Further, the first protein deposited in step (a) may be in a soluble buffer or immobilized in a gel.

15

Further, the substrate may include a plurality of microwells contained therein, and the first protein is deposited in step (a) into the microwells. In another embodiment, the substrate may include a glass plate, and the first protein array is printed onto the glass plate in step (a). According to another embodiment, the substrate

20

may include a glass plate and a plurality of gel pads on the glass plate, and the first protein is deposited in step (a) onto the gel pads. In yet another embodiment, the substrate comprises one or more filter membranes. In any event, the first protein may be deposited on the substrate

25

by a robot.

Further, the array may include an oligonucleotide, a sugar, messenger RNA and/or DNA.

30

In addition, the arrays prepared with the methodologies provided by this disclosure may include, but are not limited to, antibodies, inorganic compounds, organic compounds, peptides, peptidomimetic compounds, polypeptides or proteins, fragments or derivatives which share some or

35

all properties, e.g. fusion proteins. The components of

the arrays may be naturally occurring and obtained by purification, or may be non-naturally occurring and obtained by synthesis. Proteins, enzymes, antibodies, compositions, etc., may be obtained, when available, from commercial sources, such as Sigma (U.S.A.). Exemplary methodologies for synthesizing (for example, by using cDNA expression libraries or chemical synthesis of polypeptide followed by refolding into native proteins) or otherwise obtaining the proteins (or, for example, other polypeptides or compositions) for preparing the arrays are provided infra. Further, one skilled in the art would know that other well-known methodologies may also (or alternatively) be used.

Further, the first protein array which is deposited on the substrate need not be a single protein, but rather may be a plurality of different proteins having one or more PDZ domains. Similarly, the second protein which is applied to the protein array need not be a single protein, but rather may be a plurality of different proteins which comprise one or more amino acid sequences (S/T)-X-(V/I/L)-COOH.

Thus, the present disclosure also provides a method of preparing a protein array, comprising the steps of: (a) depositing on a substrate an array of first proteins, each first protein comprising a corresponding PDZ domain; and (b) applying a second protein, which comprises an amino acid sequence (S/T)-X-(V/I/L)-COOH, to the array of first proteins, the amino acid sequence (S/T)-X-(V/I/L)-COOH of the second protein, for each of the first proteins, binding to the PDZ domain of the first protein. Each hyphen represents a peptide bond, each parenthesis encloses amino acids which are alternatives to one other, each slash within such parentheses separates the alternative amino acids, and the X represents any amino acid which is

selected from the group comprising the twenty naturally occurring amino acids.

1062138.00602

The present disclosure also provides a method of preparing a protein array, comprising the steps of: (a) depositing on a substrate an array of a first protein, the first protein comprising a PDZ domain; and (b) applying a plurality of second proteins, each of which comprises a corresponding amino acid sequence (S/T)-X-(V/I/L)-COOH, to corresponding elements of the first protein array, for each of the second proteins, the amino acid sequence (S/T)-X-(V/I/L)-COOH of the second protein binding to the PDZ domain of the first protein in the corresponding array element. Each hyphen represents a peptide bond, each parenthesis encloses amino acids which are alternatives to one other, each slash within such parentheses separates the alternative amino acids, and the X represents any amino acid which is selected from the group comprising the twenty naturally occurring amino acids.

The present disclosure also provides a method of preparing a protein array, comprising the steps of: (a) depositing on a substrate an array of a first polypeptide, the first polypeptide comprising a PDZ domain; and (b) applying a second polypeptide which comprises an amino acid sequence (S/T)-X-(V/I/L)-COOH to the first polypeptide array, the amino acid sequence (S/T)-X-(V/I/L)-COOH of the second polypeptide binding to the PDZ domain of the first polypeptide. Each hyphen represents a peptide bond, each parenthesis encloses amino acids which are alternatives to one other, each slash within such parentheses separates the alternative amino acids, and the X represents any amino acid which is selected from the group comprising the twenty naturally occurring amino acids. Further, the array may include an oligonucleotide, a sugar, messenger RNA and/or

DNA.

The following exemplary embodiments and experimental details sections are set forth to aid in an understanding of the subject matter of this disclosure but are not intended to, and should not be construed to, limit in any way the invention as set forth in the claims which follow thereafter.

Exemplary embodiments

Some embodiments of protein array preparation are described exemplarily below, with reference to FIGS. 13A-13C and 14A-14C, to illustrate some methodologies contemplated by the subject disclosure. One skilled in the art would recognize that these methodologies (and other aspects of the disclosure described herein) may be adapted for preparing arrays that include one or more other polypeptides, oligonucleotides, sugars, messenger RNAs, DNAs, and/or other compounds.

An array of first protein spots P is deposited on a substrate (for example, a glass slide) [FIG. 13A]. A second protein (A), which comprises an amino acid sequence (S/T)-X-(V/I/L)-COOH, is applied to the first protein array [FIG. 13B]. The amino acid sequence (S/T)-X-(V/I/L)-COOH of the second protein (A) binds to the PDZ domain of the first protein (P) [FIG. 13C]. The protein array may be used to screen drugs X, Y, Z, etc.

According to one embodiment, a substrate comprising a glass plate and a plurality of microwells (FIG. 14C) formed by, for example, an enclosing hydrophobic Teflon mask may be used (see, for example, Mendoza, et al. 1999). The protein having a PDZ domain is dissolved in a buffered solution. The protein solution is then printed as an array on the

substrate through, for example, a capillary-based print head attached to a X-Y-Z robot (or another type of microdispensing liquid handling robot system). By using an automated robotic system with the open microarray, a low-cost, highly parallel assay format is feasible.

The protein may also be printed onto a treated glass slide without wells (FIG. 14A). However, use of such a substrate has the disadvantage of having higher evaporation and a greater risk of cross contamination.

According to another embodiment, gels (and gel pads) may be used to immobilize the protein having a PDZ domain. Preparation of protein microchips using gel pads is described by, for example, Arenkov, et al. 2000.

For example, according to one embodiment using gel pads, the gel pads are deposited as a micromatrix on a glass slide (FIG. 14B) and then treated according to the composition of the gel pads (for example, photopolymerization may be used for polyacrylamide gel pads). The protein, which may be dissolved in a (for example, phosphate) buffered solution, is then transferred onto the gel pads, for example, via a pin by using a robot or a manual device. Preferably, the pin and micromatrix are kept at dew point to avoid evaporation of the protein solution. Next, the protein is chemically attached to the gels. Depending on the composition of the gel, any of a number of methodologies may be used.

An advantage of using gel support for fixation of biological compounds is its large capacity for immobilized compounds. In addition, the gel pads in the array are separated from each other by a hydrophobic surface. Therefore, gel pad arrays can be used as micro-test tubes

to carry out specific interactions and chemical and enzymatic procedures with microchip substances.

5 Filter membranes also may be used to immobilize the protein. An appropriately selected membrane passively binds to, and thereby immobilizes, the protein, to produce protein filters.

10 In addition, the protein solution may be labeled by covalent linkage of a fluorescent dye to the amino groups on the protein. Fluorescence microscopy methodologies may be in conjunction with the protein-protein interaction (described exemplarily below), for example, to screen drug targets.

15 After an array of the first protein is deposited on the substrate through, for example, one of the methodologies described above, a second protein which comprises an amino acid sequence (S/T)-X-(V/I/L)-COOH is applied to the
20 micromatrix. The amino acid sequence (S/T)-X-(V/I/L)-COOH of the second protein binds to the PDZ domain of the first protein. The interaction between the first and second proteins is described below. After the second protein is applied, a wash may be applied.

25 The specific embodiments described herein are illustrative, and many variations can be introduced on these embodiments without departing from the spirit of the disclosure or from the scope of the appended claims. Elements and/or features
30 of different illustrative embodiments may be combined with each other and/or substituted for each other within the scope of this disclosure and appended claims.

35 For example, in some additional embodiments, the first protein which is deposited as an array on the substrate may

1009213.030602

comprise the amino acid sequence (S/T)-X-(V/I/L)-COOH, and the second protein which is applied to the first protein array may comprise the PDZ domain.

5 Additional variations to the embodiments described herein may be apparent to one of ordinary skill in the art from reading U.S. patent applications Serial Nos. 08/681,219 (filed July 22, 1996) and 09/230,111 (filed May 17, 1999). The contents of U.S. Serial Nos. 08/681,219 and 09/230,111
10 are hereby incorporated by reference.

Experimental methodologies and data in connection with the subject matter of this disclosure is illustrated in the Experimental Details section which follows.

15

FIRST SERIES OF EXPERIMENTS

Experimental Details

Methods and Materials

1. Screening a semi-random and random peptide library.

20 To create numerous mutations in a restricted DNA sequence, PCR mutagenesis with degenerate oligonucleotides was employed according to a protocol described elsewhere (Hill, et al. 1987). Based on the homology between human and rat, two palindromic sequences were designed for construction of
25 semi-random library. The two primers used were 5'-CGGAATTCNNNNNNNNNAACAGCNNNNNNNNNAATGAANNNCAAAGTCTGNNNT GAGGATCCTCA-3' (Seq. I.D. No. 31) and 5'-CGGAATTCGACTCAGAA NNNNNNAACTTCAGANNNNNNATCNNNNNNNNGTCTGAGGATCCTCA-3' (Seq. I.D. No. 32). Briefly, the two primers (each 200 pmol),
30 purified by HPLC, were annealed at 70 °C for 5 minutes and cooled at 23 °C for 60 minutes. A Klenow fragment (5 U) was used for filling in with a dNTP mix (final concentration, 1 mM per each dNTP) at 23°C for 60 minutes. The reaction was stopped with 1 µl of 0.5 M EDTA and the DNA was
35 purified with ethanol precipitation. The resulting

double-stranded DNA was digested with EcoRI and BamHI and re-purified by electrophoresis on non-denaturing polyacrylamide gels. The double-strand oligonucleotides were then ligated into the EcoRI-BamHI sites of the pBTM116 plasmid. The ligation mixtures were electroporated into the *E. coli* XL1-Blue MRF' (Stratagene) for the plasmid library. The large scale transformation was carried out as previously reported. The plasmid library was transformed into L40-strain cells (*MATa*, *trp1*, *leu2*, *his3*, *ade2*, *LYS2::(lexAop)⁺-HIS3*, *URA3::(lexAop)⁺-lacZ*) carrying the plasmid pVP16-31 containing a FAP-1 cDNA (Sato, et al. 1995). Clones that formed on histidine-deficient medium (*His*⁺) were transferred to plates containing 40 µg/ml X-gal to test for a blue reaction product (β -gal⁺) in plate and filter assays. The clones selected by *His*⁺ and β -gal⁺ assay were tested for further analysis. The palindromic oligonucleotide, 5'-CGGAATTC-(NNN)₄₋₁₅-TGAGGATCCTCA-3' (Seq. I.D. No. 33), was used for the construction of the random peptide library.

2. Synthesis of peptides

Peptides were automatically synthesized on an Advanced ChemTech ACT357 by analogy to published procedures (Schnorrenberg and Gerhardt, 1989). Wang resin (0.2-0.3 mmole scale) was used for each run and N^α-Fmoc protection was employed for all amino acids. Deprotection was achieved by treatment with 20% piperidine/DMF and coupling was completed using DIC/HOBt and subsequent HBTU/DIEA. After the last amino acid was coupled, the growing peptide on the resin was acetylated with Ac₂O/DMF. The peptide was cleaved from the resin with concomitant removal of all protecting groups by treating with TFA. The acetylated peptide was purified by HPLC and characterized by FAB-MS and ¹H-NMR.

3. Inhibition assay of Fas/FAP-1 binding using the C-

HFAP-10 cDNA (Sato, et al. 1995) subcloned into the Bluescript vector pSK-II (Stratagene) was in vitro-translated from an internal methionine codon in the presence of ³⁵S-L-methionine using a coupled in vitro transcription/translation system (Promega, TNT lysate) and T7 RNA polymerase. The resulting ³⁵S-labeled protein was incubated with GST-Fas fusion proteins that had been immobilized on GST-Sepharose 4B affinity beads (Pharmacia) in a buffer containing 150 mM NaCl, 50 mM Tris [pH 8.0], 5 mM DTT, 2 mM EDTA, 0.1 % NP-40, 1 mM PMSF, 50 µg/ml leupeptin, 1 mM Benzamidine, and 7 µg/ml pepstatin for 16 hours at 4 °C. After washing vigorously 4 times in the same buffer, associated proteins were recovered with the glutathione-Sepharose beads by centrifugation, eluted into boiling Laemmli buffer, and analyzed by SDS-PAGE and fluorography.

In vitro-translated [³⁵S]HFAP-1 was purified with a NAP-5 column (Pharmacia) and incubated with 3 μM of GST-fusion proteins for 16 hours at 4°C. After washing 4 times in the binding buffer, radioactivity incorporation was determined in a β counter. The percentage of binding inhibition was calculated as follows: percent inhibition = [radioactivity incorporation using GST-Fas (191-335) with peptides - radioactivity incorporation using GST-Fas (191-320) with peptides] / [radioactivity incorporation using GST-Fas (191-335) without peptides - radioactivity incorporation using GST-Fas (191-320) without peptides]. n=3.

5. Interaction of the C-terminal 3 amino acids of Fas with

FAP-1 in yeast and in vitro.

The bait plasmids, pBTM116 (LexA)-SLV, -PLV, -SLY, and -SLA, were constructed and transformed into L40-strain with pVP16-FAP-1 or -ras. Six independent clones from each transformants were picked up for the analysis of growth on histidine-deficient medium. GST-Fas, -SLV, and PLV were purified with GST-Sepharose 4B affinity beads (Pharmacia). The methods for in vitro binding are described above.

6. Immuno-precipitation of native Fas with GST-FAP-1 and inhibition of Fas/FAP-1 binding with Ac-SLV.

GST-fusion proteins with or without FAP-1 were incubated with cell extracts from Jurkat T-cells expressing Fas. The bound Fas was detected by Western analysis using anti-Fas monoclonal antibody (F22120, Transduction Laboratories). The tripeptides, Ac-SLV and Ac-SLY were used for the inhibition assay of Fas/FAP-1 binding.

7. Microinjection of Ac-SLV into the DLD-1 cell line.

DLD-1 human colon cancer cells were cultured in RPMI 1640 medium containing 10% FCS. For microinjection, cells were plated on CELLocate (Eppendorf) at 1×10^5 cells/2 ml in a 35 mm plastic culture dish and grown for 1 day. Just before microinjection, Fas monoclonal antibodies CH11 (MBL International) was added at the concentration of 500 ng/ml. All microinjection experiments were performed using an automatic microinjection system (Eppendorf transjector 5246, micro-manipulator 5171 and Femtotips) (Pantel, et al. 1995). Synthetic tripeptides were suspended in 0.1% (w/v) FITC-Dextran (Sigma)/K-PBS at the concentration of 100 mM. The samples were microinjected into the cytoplasmic region of DLD-1 cells. Sixteen to 20 hours postinjection, the cells were washed with PBS and stained with 10 µg/ml Hoechst 33342 in PBS. After incubation at 37°C for 30 minutes, the cells were photographed and the cells showing

condensed chromatin were counted as apoptotic.

8. Quantitation of apoptosis in microinjected DLD-1 cells.

For each experiment, 25-100 cells were microinjected. Apoptosis of microinjected cells was determined by assessing morphological changes of chromatin using phase contrast and fluorescence microscopy (Wang, et al., 1995; McGahon, et al., 1995). The data are means +/- S.D. for two or three independent determinations.

Discussion

In order to identify the minimal peptide stretch in the C-terminal region of the Fas receptor necessary for FAP-1 binding, an *in vitro* inhibition assay of Fas/FAP-1 binding was used using a series of synthetic peptides as well as yeast two-hybrid system peptide libraries (Figure 2A). First, semi-random libraries (based on the homology between human and rat Fas) (Figures 2B and 2C) of 15 amino acids fused to a LexA DNA binding domain were constructed and co-transformed into yeast strain L40 with pVPl6-31 (Sato, et al. 1995) that was originally isolated as FAP-1. After the selection of 200 His⁺ colonies from an initial screen of 5.0×10^6 (Johnson, et al. 1986) transformants, 100 colonies that were β -galactosidase positive were picked for further analysis. Sequence analysis of the library plasmids encoding the C-terminal 15 amino acids revealed that all of the C-termini were either valine, leucine or isoleucine residues. Second, a random library of 4-15 amino acids fused to a LexA DNA binding domain was constructed and screened according to this strategy (Figure 2D). Surprisingly, all of the third amino acid residues from the C-termini were serine, and the results of C-terminal amino acid analyses were identical to the screening of the semi-random cDNA libraries. No other significant amino acid sequences were found in these library screenings,

suggesting that the motifs of the last three amino acids (tS-X-V/L/I) are very important for the association with the third PDZ domain of FAP-1 and play a crucial role in protein-protein interaction as well as for the regulation of Fas-induced apoptosis. To further confirm whether the last three amino acids are necessary and sufficient for Fas/FAP-1 binding, plasmids of the LexA-SLV, -PLV, -PLY, -SLY, and -SLA fusion proteins were constructed and co-transformed into yeast with pVP16-FAP-1. The results showed that only LexA-SLV associated with FAP-1, whereas LexA-PLV, -PLY, -SLY, and -SLA did not (Figure 4A). In vitro binding studies using various GST-tripeptide fusions and *in vitro*-translated FAP-1 were consistent with these results (Figure 4B).

In addition to yeast two-hybrid approaches, *in vitro* inhibition assay of Fas/FAP-1 binding was also used. First, a synthetic peptide of the C-terminal 15 amino acids was tested whether it could inhibit the binding of Fas and FAP-1 *in vitro* (Figure 3A). The binding of *in vitro*-translated FAP-1 to GST-Fas was dramatically reduced and dependent on the concentration of the synthetic 15 amino acids of Fas. In contrast with these results, human PAMP peptide (Kitamura, et al. 1994) as a negative control had no effect on Fas/FAP-1 binding activity under the same biochemical conditions. Second, the effect of truncated C-terminal synthetic peptides of Fas on Fas/FAP-1 binding *in vitro* was examined. As shown in Figure 3B, only the three C-terminal amino acids (Ac-SLV) were sufficient to obtain the same level of inhibitory effect on the binding of FAP-1 to Fas as achieved with the 4-15 synthetic peptides. Furthermore, Fas/FAP-1 binding was extensively investigated using the scanned tripeptides to determine the critical amino acids residues required for inhibition (Figure 3C). The results revealed that the third amino

acids residues from the C-terminus, and the C-terminal amino acids having the strongest inhibitory effect were either serine or threonine; and either valine, leucine, or isoleucine, respectively. However, there were no differences among the second amino acid residues from the C-terminus with respect to their inhibitory effect on Fas/FAP-1 binding. These results were consistent with those of the yeast two-hybrid system (Figures 2C and 2D). Therefore, it was concluded that the C-terminal three amino acids (SLV) are critical determinants of Fas binding to the third PDZ domain of FAP-1 protein.

To further substantiate that the PDZ domain interacts with tS/T-X-V/L/I under more native conditions, GST-fused FAP-1 proteins were tested for their ability to interact with Fas expressed in Jurkat T-cells. The results revealed that the tripeptide Ac-SLV, but not Ac-SLY, abolished in a dose-dependent manner the binding activity of FAP-1 to Fas proteins extracted from Jurkat T-cells (Figures 4C and 4D). This suggests that the C-terminal amino acids tSLV are the minimum binding site for FAP-1, and that the amino acids serine and valine are critical for this physical association.

To next examine the hypothesis that the physiological association between the C-terminal three amino acids of Fas and the third PDZ domain of FAP-1 is necessary for the *in vivo* function of FAP-1 as a negative regulator of Fas-mediated signal transduction, a microinjection experiment was employed with synthetic tripeptides in a colon cancer cell line, DLD-1, which expresses both Fas and FAP-1, and is resistant to Fas-induced apoptosis. The experiments involved the direct microinjection of the synthetic tripeptides into the cytoplasmic regions of single cells and the monitoring of the physiological

response to Fas-induced apoptosis *in vivo*. The results showed that microinjection of Ac-SLV into DLD-1 cells dramatically induced apoptosis in the presence of Fas-monoclonal antibodies (CH11, 500 ng/ml) (Figures 5A, 5E and Figure 6), but that microinjection of Ac-SLY and PBS/K did not (Figures 5B, 5F and Figure 6). These results strongly support the hypothesis that the physical association of FAP-1 with the C-terminus of Fas is essential for protecting cells from Fas-induced apoptosis.

In summary, it was found that the C-terminal SLV of Fas is alone necessary and sufficient for binding to the third PDZ domain of FAP-1. Secondly, it is proposed that the new consensus motif of tS/T-X-V/L/I for such binding to the PDZ domain, instead of tS/T-X-V. It is therefore possible that FAP-1 plays important roles for the modulation of signal transduction pathways in addition to its physical interaction with Fas. Thirdly, it is demonstrated that the targeted induction of Fas-mediated apoptosis in colon cancer cells by direct microinjection of the tripeptide Ac-SLV. Further investigations including the identification of a substrate(s) of FAP-1 and structure-function analysis will provide insight to the potential therapeutic applications of Fas/FAP-1 interaction in cancer as well as provide a better understanding of the inhibitory effect of FAP-1 on Fas-mediated signal transduction.

SECOND SERIES OF EXPERIMENTS

FAP-1 was originally identified as a membrane-associated protein tyrosine phosphatase which binds to the C-terminus of Fas, and possesses six PDZ domains (also known as DHR domain or GLGF repeat). PDZ domain has recently been shown as a novel module for specific protein-protein interaction, and it appears to be important in the assembly of membrane proteins and also in linking signaling molecules in a

multi-protein complex. In recent comprehensive studies, it was found that the third PDZ domain of FAP-1 specifically recognized the sequence motif t(S/T)-X-V and interacts with the C-terminal three amino acids SLV of Fas (Fig. 9). In order to investigate the possibility that FAP-1 also interacts with the C-terminal region of p75NGFR (Fig. 8), an in vitro binding assay, was performed as well as, a yeast two-hybrid analysis by using a series of deletion mutants of p75NGFR. The results revealed that the C-terminal cytoplasmic region of p75NGFR, which is highly conserved among all species, interacts with FAP-1 (Fig. 10). Furthermore, the C-terminal three amino acids SPV of p75NGFR were necessary and sufficient for the interaction with the third PDZ domain of FAP-1 (Fig. 11A and 11B). Since FAP-1 expression was found highest in fetal brain, these findings imply that interaction of FAP-1 with p75NGFR plays an important role for signal transduction pathway via p75NGFR in neuronal cells as well as in the formation of the initial signal-transducing complex for p75NGFR.

10092138.030602

REFERENCES

1. Arenkov, P., et al. Biotechniques **27**(4): 123-31 (2000).
- 5 2. Banville, D., et al. J. Biol.Chem. **269**: 22320-22327 (1994).
3. Boldin, M.P., et al. J. Biol. Chem. **270**: 7795-7798 (1995).
- 10 4. Camerini, D., et al. J. Immunol. **147**: 3165-3169 (1991).
5. Chao, M.V. and B.L. Hempstead TINS **18**: 321-326 (1995).
- 15 6. Chinnaiyan, A.M., et al. Cell **81**: 505-512 (1995).
7. Cho, K.O., et al. Neuron **9**: 929-942 (1992).
- 20 8. Conboy, J.G., et al. J. Biol. Chem. **266**: 8273-8280 (1991).
9. Doyle, D.A., et al. Cell **85**: 1067-1076 (1996).
- 25 10. Funayama, N., et al. J. Cell Biol. **115**: 1039-1048 (1991).
11. Gould, K.L., et al. EMBO J. **8**: 4133-4142 (1989).
- 30 12. Gu, M.X., et al. Proc. Natl. Acad. Sci. U.S.A. **88**: 5867-5871 (1991).
13. Hill, D.E., et al. Meth. Enzymol. **155**, 558-568 (1987).

1000233-030602

28. Rouleau, G., et al. Nature **363**: 515-521 (1993).

29. Rowe, C.A., et al. Anal Chem. **71**(2): 433-39 (1999).
30. Sambrook, J., et al. Molecular Cloning: a laboratory manual. Second Edition. Cold Spring Harbor Laboratory Press (1989).
31. Saras, J., et al. J. Biol. Chem. **269**, 24082-24089 (1994).
32. Sato, T., et al. Science **268**: 411-415 (1995).
33. Schnorrenberg, G. and Gerhardt H. Tetrahedron **45**: 7759-7764 (1989).
34. Silzel, J.W., et al. Clin. Chem. **44**(9): 2036-43 (1998).
35. Smith, C.A., et al. Cell **73**: 1349-1360 (1993).
36. Stamenkovic, I., et al. EMBO J. **8**: 1403-1410 (1989).
37. Stanger, B.Z., et al. Cell **81**: 513-523 (1995).
38. Takahashi, T., et al. Cell **76**: 969-976 (1994).
39. Vogel, W., et al. Science **259**: 1611-1614 (1993).
40. Watanabe-Fukunaga, R., et al. Nature **356**: 314-317 (1992).
41. Wang, X.W., et al. Cancer Res. **55**: 6012-6016 (1995).
42. Westendorp, M.O., et al. Nature **375**: 497-500 (1995).

